CHARACTERISTICS OF THE IMMUNE RESPONSE OF SCOTS PINE DURING INFECTION CAUSED BY *HETEROBASIDION ANNOSUM* S.S.

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ОСОБЕННОСТИ ИМУННОГО ОТВЕТА СОСНЫ ОБЫКНОВЕННОЙ НА ИНФИЦИРОВАНИЕ *HETEROBASIDION ANNOSUM* S.S.

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В докладе освещены особенности развития инфекционного процесса в тканях саженцев сосны обыкновенной, вызванного инокуляцией их некротрофным грибом *Heterobasidion annosum* s.s. Представлены данные об экспрессии генов антимикробных пептидов – липид-переносящего белка (*Ps*LTP1) и дефензина 4 (*Ps*Def4) сосны обыкновенной в здоровых и инфицированных растениях.

Heterobasidion annosum is a causative agent of the root and butt rot and appears to be one of the most economically important conifer pathogens, which causes devastation in boreal forests at northern hemisphere. Despite biology and genetics of this fungus is well studied, the mechanisms of defense responses and resistance are still unclear, due to that investigation of response in infected pine trees is very important. A lot of data is available about plant-pathogen interaction in crops, especially it is well studied in arabidopsis. Conducting those experiments on trees is more complicated due to few disadvantages such as long lifespan, absence of host genotype in *Pinaceae* with total resistance against *Heterobasidion annosum* and also absence of avirulent strains of *H. annosum*. Lack of suitable model system make a major set back in investigation of biotic interactions in the *H. annosum* pathosystem. In this report we described the development of the infection in 3 years old pine saplings caused by root rot and evaluated the changes in transcription level of the antimicrobial proteins: lipid transfer protein 1 (*PsLTP1*) and defensin 4 (*PsDef4*), which supposedly are related to the development of defense response.

Material and methods. 3 years old pine saplings were inoculated with *H. annosum*. For this purpose the blocks of agar (5x5x2 mm^3) containing mycelium of *H. annosum* were transferred on the cut of bark (5x8 mm^2), made 6 cm above the root collar. The control was made in the same manner but without the mycelium. The contact zone was covered with parafilm and left for the infection to develop from April till September, after that the samples were collected and the cuts were made from inoculation zone and 2 cm above the place of contact, to evaluate the development of the infection using light microscopy. In other experiment the expression levels of antimicrobial peptides were checked in the roots of 80 years old Scots pine trees, both infected and not infected. The levels of gene expression were checked on the mRNA level using a PCR technique. mRNA was obtained using modified method of lithium-chloride precipitation by Chang (Chang, 1993). Primer pairs were picked up according to the sequences recently deposited by us in GenBank: *PsLTP1* (Acc.No. JN980402.1) and *PsDef4* (Acc.No. KJ601732.1). As a control for the reaction, we chose the house-keeping gene *RPL44* (Acc.No. EL342388.1), which also was used for the calculation of the relative values of the expression level. PCR was run for 35 cycles in a thermal cycler using the program: 95^{O}C, 1 min; 54^{O}C, 1 min, 72^{O}C, 1 min. The PCR products were electrophoresized on a 1.5% agarose gel and visualized with ethidium bromide staining. Densitometric analysis was run with Software GelProAnalyzed 4.0.

In wood trees the first line of defense towards different pathogens is presented by bark. Mechanical barrier of the strong lignified walls together with chemical properties of the phenolic compounds create multifunctional blockage for pathogens. Anyway, even tiny lesions on the bark can serve as a doorway for infection, especially if the last one is caused by necrotrophic pathogen such as root sponge, which appears to be very aggressive wood destroyer. As we know, the mode of spreading of the mycelium depends from many factors and one of them is the season at which the pathological process is developing.

On the Fig. 1 we can observe the spreading of the necrosis during vegetation period to the heartwood of the sapling and its localization at sapwood of the previous year.

Due to intensive proliferation of the cambium, the layer of sapwood with increased quantity of the resinoids filled with resin was formed, restricting the spreading of the mycelium onto the primary heartwood in the current year. On the radial cuts we observed intensive secretion of the resin around the wound, followed by formation of cone-like necrosis zone from the bark to the heartwood. On the radial cut of infected sapling we can observe line of necrosis (brown), which spreads as a trace in a heartwood, forming resinoids. In a sapwood of control plants, which were only wounded, formation of resinoids wasn't observed.

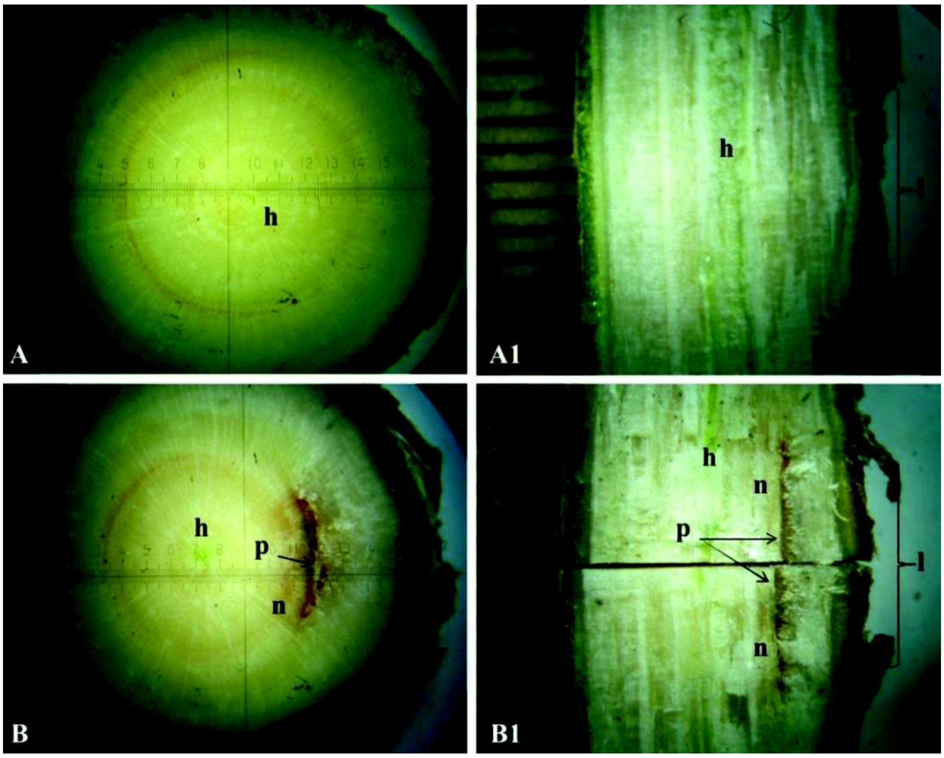

Figure 1. Spreading of the mycelium of *H.annosum* in wood of 3 years old saplings of Scots pine during vegetation period

A, B – cross-cuts through the center of inoculation of the infected and control saplings, respectively; A1, B1 – radial cuts of the same samplings. p – resin pocket; l – line of bark cut; n – necrotic zone; h – heartwood. Cut thickness 1-1.5 mm (Magnification 32x. Division 1=0.5 mm).

Besides we have investigated the expression levels of the two of antimicrobial peptides, which supposedly are involved in plant defense reactions - *PsLTP1* and *PsDef4*. Results are presented on a Fig.2.

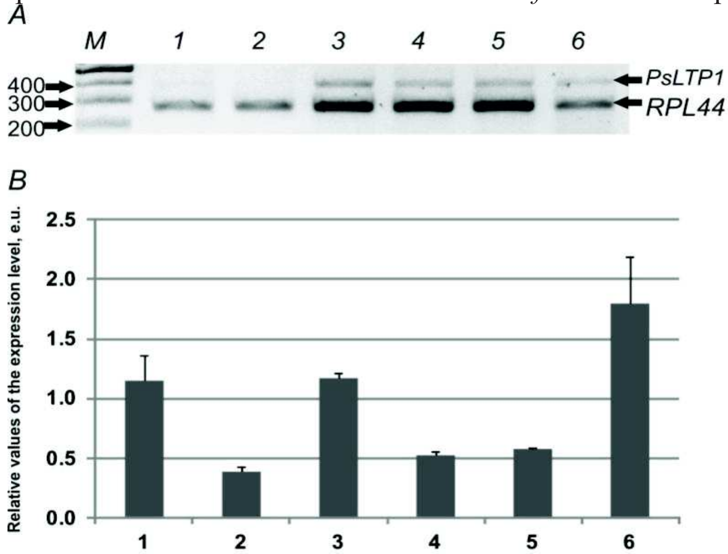

Figure 2. Analysis of *PsLTP1* expression in the Scots pine tissues of 3 years old saplings (1-4) and 80 years old trees (5, 6).

A – electrophoregram of the RT-PCR products obtained from RNA of 3 years old pine saplings inoculated with mycelium of H. annosum (2 – from the zone of inoculation and 4- 2 cm above), control samples were obtained from the saplings inoculated without mycelium (1 – from the zone of inoculation and 3–2 cm above). 5, 6 – samples obtained from 80 years old Scots pine trees healthy and naturally infected with root sponge, respectively. M - GeneRuler 100 bp Plus DNA Ladder (Fermentas). Right arrows indicate the PCR-products: *PsLTP1* and "house-keeping" gene *RPL44*. B – the values of the expression level of *PsLTP1* calculated relative to *RPL44*

As we see, both, control samples from the zone of inoculation and 2 cm above showed the same level of expression of PsLTP1. In case of inoculation with the mycelium of root sponge we observed decreasing of the expression level of PsLTP1 three times compared to the appropriate control sample. In case of the roots of 80 years old Scots pine we obtained an opposite results, where the level of transcripts of PsLTP1 in infected organs was three times higher compared to the not infected plants. On parallel we have performed the same set of experiment checking the expression level of another gene PsDef4, but we haven't seen any transcripts, so we suppose that expression of this gene requires specific conditions or factors which induce the expression of this gene.

Taking all obtained data together we can conclude that during artificial infection, defense mechanisms relay on the resin secretion on the wounded area and, if infection occurred naturally, plant develop specific defense response, where antimicrobial peptides are involved. This could be explained with evolutionary-established host-pathogen interactions.

АНАМОРФНЫЕ ГРИБЫ НА ХВОЙНЫХ РОССИЙСКОГО ДАЛЬНЕГО ВОСТОКА

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ANAMORPHIC FUNGI ON CONIFERS IN THE RUSSIAN FAR EAST

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At present the species composition of anamorphic fungi that inhabit the conifers in the Russian Far East consists of 90 species belonging to 62 genera from classes *Hyphomycetes* (65 species from 40 genera) and *Coelomycetes* (25 species from 22 genera). These fungi are associated with 16 species of conifers from such genera as *Abies*, *Larix*, *Picea*, *Pinus*. 30 species of anamorphic fungi have been found on needles of conifers, 22 species – on coniferous twigs, 20 – on wood and bark. The group of soil micromycetes (25 species) causing the root rot diseases have been isolated from coniferous seedlings.

Хвойные леса, занимающие почти ¾ территории российского Дальнего Востока, представлены светлохвойными лиственничными, темнохвойными елово-пихтовыми и хвойно-широколиственными формациями. Главнейшими эдификаторами в темнохвойных лесах являются ель аянская (*Picea ajanensis*) и пихта белокорая (*Abies nephrolepis*), в хвойно-широколиственных – сосна кедровая корейская (*Pinus koraiensis*) и дуб монгольский (*Quercus mongolica*), в светлохвойных – лиственница Гмелина (*Larix gmelinii*). Своеобразные стелющиеся леса-заросли кедрового стланика (*Pinus pumila*) характерны для берингийской лесотундровой области и заносимых снегами склонов гор. В почвенном покрове лиственничных лесов материковой части региона преобладают мерзлотно-таежные светлоземы и перегнойно-карбонатные почвы, в елово-пихтовых лесах – буротаежные, в хвойно-широколиственных – бурые лесные почвы. На полуострове Камчатка и северных Курильских островах (Шумшу и Парамушир) распространены дерновые почвы, на острове Сахалин и Южных Курильских островах (Итуруп, Кунашир, Шикотан) – почвы подзолистого типа.

По результатам микологического мониторинга на Дальнем Востоке России обнаружено более 500 видов грибов из различных систематических групп (*Zygomycota*, *Basidiomycota*, *Ascomycota*, *Anamorphic fungi*), развивающихся на хвойных древесных породах или ассоциированных с ними. Микромицеты, входящие в состав морфологической группы *Anamorphic fungi*, включают в себя представителей классов *Hyphomycetes* и *Coelomycetes*, обитающих на хвое, ветвях, коре, древесине, опаде и валеже. Более 200 видов микромицетов выделено из почв хвойных лесов. Грибы сапротрофы, к которым принадлежат большинство выявленных на хвойных региона анаморфных грибов, остаются до настоящего времени одной из самых слабоизученных групп.

Сбор образцов анаморфных грибов на хвойных древесных породах проводился, начиная с 1970 г., но особенно интенсивно – в период 2003-2006 гг. в рамках выполнения проекта «Микобиота хвойных Дальнего Востока России» Программы ОБН РАН «Биологические ресур-